Brain death: from inflammation to metabolic changes
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

Link to publication in University of Groningen/UMCG research database

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METABOLIC CHANGES IN BRAIN DEAD RATS

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\textit{In preparation}
ABSTRACT

Introduction: Grafts from brain dead donors have worse long term outcome compared to living donors. Brain death can be understood as a complex disturbance in body homeostasis resulting in hemodynamic instability, hormonal impairment and inflammation. These three factors can influence cellular metabolism. Our aim is to study metabolic changes in brain dead rats and their influence in kidney and liver tissue quality.

Methods: Brain death was induced in mechanically ventilated rats by inflation of a Fogarty catheter in the epidural space. After four hours of BD, plasma, kidney and liver tissue were collected. RT-qPCR, routine biochemistry and immunohistochemistry were performed.

Results: After BD period an increase in lactate and urea while a decrease in glucose plasma levels were found in brain dead animals. An increase in medium and long chain Acylcarnitine plasma levels was found in brain dead animals compared to controls. A decrease in hepatic glycogen levels was found in brain dead animals and a down-regulation of gluconeogenesis related enzymes in kidney and liver but an up-regulation of lactate dehydrogenase in kidney and not in the liver of brain dead animals compared to sham operated animals.

Conclusion: Important metabolic changes occur during brain death. An increase in other energy supplies utilization as fatty acid and proteins seems to be happening. Apparently, in a BD situation the kidney is increasing anaerobic metabolism while liver is not.
INTRODUCTION

The shortage of organs, suitable for transplantation, remains one of the major problems in the field. Extended criteria and deceased cardiac dead (DCD) donors are common strategies used to address this problem. Long and short term outcomes of grafts obtained from these sources are inferior compared to living donation [4, 47]. However brain death donation represent the largest pool of organ donors. Interestingly grafts from BD donors have worse outcomes compared to HLA mismatched living donors [43, 45, 46].

Brain death is caused by a significant and progressive cerebral ischemia. The resulting injury will lead to cerebral oedema and intracranial hypertension, thus a decrease in cerebral perfusion pressure and consequently more cerebral ischemia. Cushing described the sympathetic response to the intracranial hypertension which is also called catecholamines storm. This sudden increase in circulating vasopressors will elevate blood pressure and can be considered as the starting point of the typical hemodynamic pattern in brain death donors. This pattern will finish with spinal cord ischemia and the loss of the sympathetic tone in the peripheral vascular bed triggering the vascular collapse at the end of the brain death period [2, 13, 21, 25, 32, 48].

Brain stem ischemia triggers hormonal dysregulation. Pituitary function is affected and plasma levels of Cortisol, thyroid hormones (T₃/T₄), Insulin and Antidiuretic Hormone (ADH) decrease below baseline levels after brain death has been established [6, 31, 32]. Hormonal therapy is still matter of debate [11, 12, 16, 24, 26, 38]. However, in previous experiments we showed that pre-treatment of T₃ thyroid hormone can reduce apoptosis in liver tissue of brain dead rats.

Brain death is characterized by a systemic inflammatory response [1, 18, 27, 29] Circulating cytokines levels rise, including interleukin-6 (IL-6), interleukin-10 (IL-10), Tumor Necrosis Factor-alpha (TNF-α), Transforming Growth Factor-beta (TGF-β), and monocyte chemotactic protein 1 (MCP-1) [27, 28, 46]. This inflammatory environment permits an influx of inflammatory cells in kidneys, liver, and lungs, which triggers a local inflammatory and apoptotic response [18, 29]. The origin of this inflammatory status is not well understood, but there are reasons to assign a role to cerebral inflammatory cytokines, complement activation [7, 8] and to increased intestinal permeability [22]. Our previous studies shown that anti-inflammatory effects of prednisolone did not improve kidney nor liver quality of brain dead rats measured by creatinine plasma levels, hepatic cellular injury markers as ASAT and ALAT and in both tissues the relative expression of pro-apoptotic proteins [36, 37].

Based on this knowledge, we postulate that hemodynamic, hormonal and inflammatory changes are affecting organ metabolism during brain death. In severe sepsis these three factors are related to cytopathic hypoxia, which means a normal oxygen delivery but poor oxygen utilization plus an increase in lactate plasma levels and impairment of pyruvate dehydrogenase [15]. Lee et al. [23] summarize evidence about metabolic changes in sepsis, mainly mitochondrial dys- function: a decrease in oxidative phosphorylation and
hepatic ATP content during sepsis. Few articles have been written about this specific topic in brain death. Novitzky et al. [30] reported in a baboon model of brain death using carbon labeled compounds that brain dead animals had an increased anaerobic metabolism accompanied by a deterioration of the hemodynamic status. Sztark et al. [40] have shown in muscle biopsies from brain dead human donors that mitochondrial function was affected when compared to healthy volunteers. The conversion to anaerobic metabolism could be explained by two different reasons: tissue hypoxia or mitochondrial dysfunction. This point can be crucial when considering donor treatment in order to improve organ quality for transplantation.

The aim of this article was to summarize metabolic changes in brain dead animals focusing on liver and kidney.

Methods Animals
Male adult Fisher F344 rats (270-300 g) were used. All animals received care in compliance with the guidelines of the local animal ethics committee according to Experiments on Animals Act (1996) issued by the Ministry of Public Health, Welfare and Sports of the Netherlands. Animals were anaesthetized using isoflurane with 100% O₂. Animals were intubated via a tracheostomy and ventilated (Tidal Volume: 6.5 ml/BW(Kg) ml per stroke, PEEP of 3 cm of H₂O initial respiratory rate of 120 and corrected base on ETCO₂) throughout the experiment. Two cannulas were inserted in the femoral artery and vein for continuous mean arterial pressure (MAP) monitoring and volume replacement. Through a frontolateral hole drilled in the skull, a no. 4 Fogarty catheter (Edwards Lifesciences Co, Irvine, CA) was placed in the epidural space and inflated with saline using a syringe pump (Terufusion, Termo Co., Tokyo, Japan). BD induction was achieved by inflating the catheter at a speed of 16 ml/hr. Balloon inflation was terminated once a rise in the MAP above 80mmHg was noted; reflecting the autonomic storm at the beginning of BD. Induction was confirmed by the absence of corneal reflexes. Following confirmation of BD, ventilation was continued and anaesthesia was terminated. Mean arterial pressure (MAP) was maintained above 80 mmHg. If necessary, colloid infusion with polyhydroxyethyl starch (HAES) 10% (Fresenius Kabi AG, Bad Homburg, Germany) was started (at a maximum rate of 1ml/hr) to maintain a normotensive MAP. Unresponsiveness to HAES administration indicated the start of an intravenous noradrenaline (NA) drip (1mg/mL). A homeothermic blanket control system was used throughout the experiment. After the experimental time, blood and urine were collected, after which all abdominal organs were flushed with cold saline. After the flush- out, organs were harvested and tissue samples were snap frozen in liquid nitrogen and stored at -80 °C or fixated in 4% paraformaldehyde. Plasma samples and urine were also snap-frozen and stored. Animals in the Sham operated group followed the same surgical procedure but without the introduction of the epidural Fogarty catheter. For the control group, blood, urine and tissue was collected under anesthesia without any extra surgical procedure.
Animals were randomly assigned to one of the following experimental groups \((n=8)\):

1. Brain Death
2. Sham Operated
3. Control Living Donor

**Plasma Determinations**

Plasma levels of glucose, lactate and urea were determined at the clinical chemistry lab of University Medical Centre Groningen according to standard procedures.

**Determination of acylcarnitine concentrations in plasma:**

For determination of acylcarnitine concentrations, 10 μl of plasma was mixed with 100 μl of acetonitrile and 100 μl of methanol–water \((80:20 \text{ v/v})\) containing internal standards \(1,1,1\text{-N-methyl-}^{2}\text{H}_3\text{-L-carnitine, } [2^\text{H}3]\text{acetyl-L-carnitine, } [3,3,3^2\text{H}_1]\text{propionyl-L-carnitine, } [8,8,8^2\text{H}_3]\text{octanoyl-L-carnitine, } [10,10,10^2\text{H}_2]\text{decanoyl-L-carnitine and } [16,16,16^2\text{H}_3]\text{hexadecanoyl-L-carnitine (VU Medical Center, Amsterdam, The Netherlands), vortexed and centrifuged at 14000 g for 10 min at 4 °C. Concentrations of acylcarnitines were measured in the supernatant with an API 3000 LC-MS/MS equipped with a Turbo ion spray source (Applied Biosystems/MDS Sciex, Ontario, Canada) as previously described [10].}

**Liver Glycerogen estimation**

Glycerogen tissue liver concentration was estimated in two different fashions. First periodic acid–Schiff (PAS) staining was performed in paraffin embedded liver samples. Then all slices were scanned and 10 pictures per slice at 20x of magnification were used. The positive PAS area was estimated using ImageJ script design for this purpose [34]. The final value per slice was the mean of those ten positive areas. Second, following the manufacturer's instructions a Glycerogen assay kit (MAK016, Sigma-Aldrich Co. 3050 Spruce St. St. Louis, MO 63103, US.) was performed to quantify glycerogen content.

**RNA isolation and cDNA synthesis**

Total RNA was isolated from whole liver sections by using TRIzol (Life Technologies, Gaithersburg, MD). RNA samples were verified for absence of genomic DNA contamination by performing RT-PCR reactions in which the addition of reverse transcriptase was omitted, using GAPDH primers. For cDNA synthesis, 1 μl T11VN Oligo-dT \((0.5 \text{ μg/μl})\) and 1μg mRNA were incubated for 10 min at 70 °C and cooled directly after that. cDNA was synthesized by adding a mixture containing 0.5 μl RNaseOUT® Ribonuclease inhibitor (Invitrogen, Carlsbad, USA), 0.5μl RNase water (Promega), 4 μl 5 x first strand buffer (Invitrogen), 2 μl DTT (Invitrogen), 1 μl dNTP's and 1μl M-MLV reverse transcriptase (Invitrogen, 200U).
The mixture was held at 37 °C for 50 min. Next, reverse-transcriptase was inactivated by incubating the mixture for 15 min at 70 °C. Samples were stored at – 20 °C.

Real-Time PCR

Fragments of several genes were amplified with the primer sets outlined in Table 7.1. Pooled cDNA obtained from brain-dead rats were used as internal references. Gene expression was normalized with the mean of β-actin mRNA content. Real-Time PCR was carried out in reaction volumes of 15μl containing 10μl of SYBR Green mastermix (Applied biosystems, Foster City, USA), 0.4μl of each primer (50μM), 4.2μl of nuclease free water and 10 ng of cDNA. All samples were analyzed in triplicate. Thermal cycling was performed on the Taqman Applied Biosystems 7900HT Real Time PCR System with a hot start for 2 min at 50 °C followed by 10 min 95 °C. Second stage was started with 15 s at 95 °C (denaturation step) and 60 s at 60 °C (annealing step and DNA synthesis). The latter stage was repeated 40 times. Stage 3 was included to detect formation of primer dimers (melting curve) and begins with 15 s at 95 °C followed by 60 s at 60° C and 15 s at 95 °C. Primers were designed with Primer Express software (Applied Biosystems) and primer efficiencies were tested by a standard curve for the primer pair resulting from the amplification of serially diluted cDNA samples (10 ng, 5 ng, 2.5 ng, 1.25 ng and 0.625 ng) obtained from brain-dead rats. PCR efficiency was found to be 1.8 < ε < 2.0. Real-time PCR products were checked for product specificity on a 1.5% agarose gel. Results were expressed as $2^{-\Delta\Delta CT}$ (CT: Threshold Cycle).

Table 6.1: Primer sequences used for Real-Time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCK-1</td>
<td>5’-TGTTTCCCGAGGTTGCATCT-3’&lt;br&gt;5’-CTGCTACAGCTAAGCTGAAAGCTG-3’</td>
<td>91</td>
</tr>
<tr>
<td>PK</td>
<td>5’-TGAGGACTGTGGAAGCTGACTG-3’&lt;br&gt;5’-CTTTATTCAATGCCCTCTCTCCTCCTCCTCCTCCTCCTC-3’</td>
<td>81</td>
</tr>
<tr>
<td>LDHA</td>
<td>5’-AATATTACGTGAAATGTAAGATCTGACATG-3’&lt;br&gt;5‘-TTTCCCTTGCCGGCATGACACTTGAG-3’</td>
<td>70</td>
</tr>
<tr>
<td>PC</td>
<td>5’-ATCTCTTGGCTACAATAAGGTGTGTCATG-3’&lt;br&gt;5’-CAGAGGTAAACCCCTCCTCCCA-3’</td>
<td>88</td>
</tr>
<tr>
<td>PFK-1</td>
<td>5’-GCATAGACAGGTTCTGAGCTA-3’&lt;br&gt;5’-AGCAGTGGGAGGAGAGAGAT-3’</td>
<td>74</td>
</tr>
</tbody>
</table>
Hepatic concentration of adenosine-5′-triphosphate (ATP)

Frozen liver tissue was cut into 20 mm slices and a total amount of 650 mg was homogenized in 1 mL of SONOP (0.372 g EDTA in 130 mL H₂O and NaOH (ph 10.9)+370 mL 96% ethanol) and sonificated. Precipitate was removed by centrifugation (13,000 rcf for 10 min). Supernatant was diluted with SONOP to attain a protein concentration of 200–300 mg/mL (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL) and mixed with 450 mL of 100 mM phosphate buffer (Merck; ph 7.6–8.0). Fifty microliters of phosphate buffered supernatant was used for ATP measurement using ATP Biololinescence assay kit CLS II (Boehringer, Mannheim, Germany) and a luminometer (Victor3 1420 multilabel counter, PerkinElmer). ATP concentrations were calculated from a calibration curve constructed on the same plate, corrected for amount of protein, and values were expressed as mmol/g protein.

Statistical analysis

For statistical analysis between groups, the Mann-Whitney test was performed, with p < 0.05 regarded as significant. Results are presented as mean ± SD (standard deviation). Statistical analyses were performed using Prism 5.0. (GraphPad Software. CA. USA)

RESULTS

We measured glucose plasma levels in order to estimate one of the available energy sources. There was no difference between Control and Sham group (11.2 ± 0.42 mmol/L, 10.8 ± 0.62 mmol/L, p = 0.4643). Brain Death group had lower glucose levels when compared to Control or Sham groups (9.27 ± 0.86 mmol/L, p = 0.0357 and p = 0.0108, respectively). Brain Death group had a higher Lactate plasma levels when compared with Control or Sham groups (2.6 ± 0.43 mmol/L, p = 0.0476, p = 0.0159, respectively) No difference was found between Control and Sham animals (1.35 ± 0.35 mmol/L, 1.4 ± 0.46 mmol/L, p = 0.8095.) In the case of Urea plasma levels, Brain Dead animals had a higher concentration when compared with Control or Sham animals (19.6 ± 2.76 mmol/L, p = 0.0002 in both cases). This group had a higher concentration of Urea in plasma when compared to Control animals (10.3 ± 0.46 mmol/L and 8.43 ± 0.7 mmol/L, respectively. p = 0.0005. Figure 6.1)

Acylcarnitine plasma concentrations were determined in Control and Brain Death groups. Long Chain Acylcarnitine (LCAC) concentration was found higher in Brain Dead animals compared to Control animals (0.388 ± 0.0627 and 0.244 ± 0.0995 μmol/L, p = 0.0140). Medium Chain Acylcarnitine (MCAC) concentration was higher in Brain Death group compared Control group (0.154 ± 0.0192 and 0.124 ± 0.0113. p = 0.0028. Figure 6.2)

Glycogen liver storage was assessed by PAS staining and molecular quantification. In the first case, the positive area for PAS staining was lower in Brain Dead compared
Sham operated animals (8.80 ± 6.81 and 44.8 ± 23.3 positive area percent. p = 0.0047)
The enzymatic assay has shown a similar result. Glycogen liver content was lower in Brain Dead animals compared with Sham operated animals. (0.054 ± 0.017 and 0.154 ± 0.067 mg/g of liver. p = 0.0286. Figure 6.3)

The relative expression of Glycolysis and Gluconeogenesis related genes was measured in the liver and kidney in Sham and Brain Death groups. Glycolysis was assessed by measuring the relative expression of two key enzymes in the pathway. Relative Phosphofructokinase-1 (PFK-1) expression was reduced in Brain Death group compared to Sham group in the Kidney (1.84 ± 0.44. and 2.52 ± 0.52. p = 0.0207) but did not change in the liver (1.32 ± 0.37 and 1.30 ± 0.19. p = 0.7768). Relative Pyruvate Kinase (PK) expression did not change in the liver (1.73 ± 0.45 and 2.25 ± 1.06. p = 0.0827) nor kidney (4.04 ± 1.20 and 3.23 ± 1.51 p = 0.0718) between Sham and Brain Death groups. Figure 6.4.

Gluconeogenesis related genes were studied by measuring relative expression of PEP carboxykinase-1 (PCK-1) in liver (0.12 ± 0.07 and 2.51 ± 0.61. p = 0.0002) and kidney tissue (0.85 ± 0.44 and 5.26 ± 1.0. p = 0.0003), in both cases the relative expression was decreased in Brain Death compared with Sham group. The second studied gene in this pathway was Pyruvate Carboxylase (PC). The relative PC expression in the kidney did not change between Brain Dead and Sham groups (1.29 ± 0.34 and 1.46 ± 0.27. p = 0.2281). In the case of the liver there was a decrease in the relative expression of PC in Brain Death group compared Sham group (1.06 ± 0.28 and 1.73 ± 0.33. p = 0.0006. Figure 6.5)

In order to assess increased anaerobic metabolism we measured relative Lactate dehydrogenase A (LDHA) expression. In the liver we did not find a difference between Brain Death and Sham groups (1.40 ± 0.54 and 1.85 ± 0.62. p = 0.3754). In the case of the kidney there was an increase in the relative LDHA expression in Brain Death compared Sham group (8.30 ± 1.98 and 4.74 ± 0.97. p = 0.0006. Figure 6.5)

Hepatic concentration of adenosine-5'-triphosphate (ATP) was measured in Brain Death and Control group. No difference was found using this technique (31.4 ± 15.8 and 22.3 ± 8.61. p = 0.2942)

DISCUSSION

Changes in energy metabolism will determine cell survival; a negative energy balance will stop cell cycle [14] and could trigger autophagy or apoptosis [20, 42, 44], and lead to cellular death. Silva et al. [39] found, using a microdialysis technique, that liver grafts with initial poor function (IPF) and histological evidence of moderate to severe or severe ischemia-reperfusion injury had higher extracellular lactate levels compared with non IPF liver grafts. Energy homeostasis is probably one of the most tightly regulated functions in the entire body. Energy balance seems to be an important parameter to assess graft quality. During brain death hormonal regulation is lost and many of those regulatory
functions are uncontrolled [9, 31]. In this article we describe metabolic changes in brain
dead rats in order to understand changes in energy balance.

We found a decrease in glucose plasma levels but also a decrease in glucose
storage as hepatic glycogen in brain dead animals was lower. These results together
can be interpreted as a depletion of carbohydrate energy source during brain death.
We hypothesize that this depletion could be related with a lower liver production and
increased glucose oxidation. The down-regulation of gluconeogenic enzymes supports
this theory.

Lactate plasma levels are increasing in brain dead rats, as has been described by others
[33, 35, 39]. A similar situation has been found in other animal models and humans
[17, 30, 40]. We can interpret this as an increase in lactate production by anaerobic
metabolism and/or a decrease in lactate clearance expressed by a reduction in the Cori
cycle activity [19]. Interestingly, a similar result was found by Tapia et al. [41] in a model of
septic shock, this decrease in lactate clearance is not related with hepatic hypoperfusion
or hypoxia. In our model blood pressure is kept above 80 mmHg with normal oxygen
saturation, we assume that renal hypoxia and reduced hepatic lactate clearance explains
this increase in plasma levels of lactate in BD animals.

Our results suggest a metabolic shift from glucose oxidation to fatty acid oxidation.
Long chain and medium chain acylcarnitine metabolites are increasing during brain
death. Probably because of a decreased carbohydrate availability and a higher energy
demand attributable to catecholamine release and the inflammatory response as a part
of the brain death [3].

An alternative energy source can be found in amino acids, although it usually one of
the last utilized energy sources. Amino acid deamination produce urea in the urea cycle
and also pyruvate, oxaloacetate, fumarate, succinyl-CoA or Acetyl-CoA can be formed
to supply the citrate cycle and oxidative phosphorylation in the mitochondria or lactate
formation due to fermentation. We found an increase in urea plasma levels which could
be related with amino acid catabolism, but our animals had also increased creatinine
plasma levels, which could, by it self, explain urea levels.

Interestingly ATP content in liver tissue is not changing due to brain death, which
means that oxidative phosphorylation or fermentation activity is still able to maintain
normal levels of ATP, despite glucose storage depletion and shift to alternatives
energy sources.

We hypothesize that these changes in metabolism are probably linked with tissue
hypoxia and/or mitochondrial dysfunction. However, we think that these two phenomena
are affecting liver and kidneys with different intensity. Based on lactate dehydrogenase
expression, it seems that the kidney prefers anaerobic metabolism while the liver is not.
Using these data we cannot conclude if this is related to a decrease in oxygen delivery or
a primary mitochondrial dysfunction.
These results together show that during BD there is complex metabolic disturbance characterized by glycogen and glucose decrease, fatty acid oxidation, increased energy demand and increase in anaerobic metabolism. Cankayali et al. [5] shown that brain dead donors had neither thermogenic response nor change of energy expenditure when parenteral nutrition was administered, since these are hypothalamic depending reactions. The lost of hormonal control may be part of the mechanism for this metabolic disturbance.
REFERENCES


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FIGURES

Figure 6.1: Plasma Levels. Glucose, Lactate and Urea in Control, Sham-operated and Brain Dead animals

Figure 6.2: Acylcarnitine concentrations in Control and Brain Death groups
Figure 6.3: Glycogen liver estimation
Figure 6.4: Relative expression of Glycolysis related enzymes in the liver and kidney.
Figure 6.5: Relative expression of Gluconeogenesis related enzymes in the liver and kidney.

\* p < 0.05